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THE INHIBITION OF OESTRUS BY EX-
TRACTS OF THE ANTERIOR LOBE
OF THE PITUITARY BODY

A DISSERTATION SUBMITTED TO THE
GRADUATE FACULTY IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY AND PHARMACOLOGY

By
MARIE C. D'AMOUR

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THE INHIBITION OF OESTRUS BY EXTRACTS OF THE ANTERIOR LOBE OF THE PITUITARY BODY¹

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The first account of oestrus inhibition and abnormal formation of luteal tissue following the parenteral administration, to rats, of extracts of the anterior pituitary, was made by Evans and Long (1, 2) more than a decade ago. These experiments have been confirmed and extended by a number of investigators who demonstrated that anterior pituitary extracts cause the formation of functional corpora lutea inasmuch as placentomata were more readily produced in the rat if anterior pituitary extract was properly administered (3, 4). A lengthening of the period of gestation in the rat was produced by Teel (5) by administering a similar extract presumably because of the abnormal persistence of functional corpora lutea. The conclusions that oestrus inhibition, as a result of the administration of anterior lobe extract, is an indirect effect and that it is due to the abnormal formation and persistence of corpora lutea, are more or less generally accepted.

This paper is a report of an investigation of the oestrus inhibiting properties of extracts of bovine anterior pituitary tissue and of whole sheep pituitary bodies. Extracts of tissues other than the pituitary have also been made and assayed. In the case of anterior pituitary tissue some data have been gathered as to the best extraction procedure. In comparing beef and sheep pituitary extracts in respect to their oestrus inhibiting (in the adult animal) and gonad stimulating (in the immature animal) properties, no evidence for separate luteinizing and follicle stimulating

¹ This investigation was aided in part by a grant to the University of Chicago by the Rockefeller Foundation.

principles was obtained. Some data on the feasibility of quantitative assay are presented.

THE NORMAL OESTROUS CYCLE

Young mature female rats were used throughout this study. It was necessary, first of all, to determine the length and variability of the oestrous cycle inasmuch as the lengthening of the

TABLE 1
The normal oestrous cycle in the rat

LENGTH	NUMBER OF INSTANCES	
	Long and Evans	Our series
<i>days</i>		
3	65	38
4	789	479
5	634	107
6	233	29
7	69	6
8	60	2
9	30	2
10	24	3
11	16	
12	22	3
13		2
17		2
19		1
Total	1,942	674
Mean length of oestrous cycle	5.06 $\pm$ 1.58*	4.39 $\pm$ 1.40

* Mean and standard deviation calculated without including cycles longer than twelve days. Detailed data on the longer cycles are not available.

cycle was usually taken as evidence that a given extract had caused the abnormal formation of luteal tissue in the ovary. Vaginal smears of 112 normal rats were therefore made once daily; all the cycles occurring within a period of a little more than four weeks were recorded.² These data, on 674 cycles, are compared with those of Long and Evans (6) in table 1.

² In this and all subsequent experiments, the vaginal smears were made at approximately the same time once each day. The diet, time of feeding, time of cleaning cages, etc., were kept as uniform as possible.

The determination of the significance of the mean oestrous cycles of treated animals presents some difficulties inasmuch as the comparisons are usually made between the mean of the large normal series (674 oestrous cycles) and a small experimental series.^{3,4} t in the sense in which Fisher (7) uses the term is calculated as

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}}}$$

in which $\bar{x}$ = a mean, σ = a standard deviation and n = the number of cycles. If the term $\frac{\sigma_2^2}{n_2}$ is taken as that of the normal series it may, in some cases, be negligibly small.

The probability that a given $\bar{x}_1$ differed significantly from $\bar{x}_2$, the mean of the normal series, was obtained from Fisher's table of t ; the number of degrees of freedom were assumed to be those of the smaller experimental series.

An arbitrary procedure, based upon preliminary experiments with oestrus inhibiting extracts known to be active, was utilized in assigning a given cycle to a given experimental period. Before every course of injections, each animal was usually required to exhibit two or more preliminary cycles of normal length. Every cycle occurring (a) between the third day of injection and the last day of the injection period, and (b) during the injection period but not terminating until after the injections had ceased, was regarded as belonging to the injection period. The cycle was counted from the last occurring in the given control period if none appeared while injections were being made. The mean cycle lengths in the subsequent tables were computed from such a treatment of the data.

EXTRACTS OF BOVINE ANTERIOR PITUITARY GLANDS

Evans (8) was able to extract the growth promoting, and also, apparently, the luteinizing principles by aqueous media of acid,

³ We are indebted to Prof. Sewall Wright for aid in the statistical treatment of the data.

⁴ In table 3, B, control and experimental mean oestrous cycles were compared.

neutral or alkaline reaction. Similar reports have been made by other investigators. We have consistently found that extraction at an alkaline pH yields a more potent final product, as tested by its ability to cause oestrus inhibition, than does extraction carried out at pH 7.0 or less. When, therefore, a comparison was made between fresh and acetone dessicated beef anterior lobe, extraction was performed at an alkaline pH. Such a comparison was worth while because, if one could employ the acetone dessicated material, he would obtain a final product containing a lower concentration of solids. Moreover the acetone desiccated powder could be readily stored, probably without loss of activity, and could serve as a more uniform source of the hormone studied.

Fresh beef anterior lobes were dissected free from extraneous tissue and ground in a food chopper. One portion was extracted directly. A second portion was subjected to acetone treatment. Twelve volumes of acetone were added to this second portion and allowed to extract with frequent stirring at room temperature for four or five hours. The acetone was decanted and saved. Twelve volumes of fresh acetone were added to the residue and the suspension reextracted in the icebox overnight. After the acetone was decanted, the residue was dried over phosphorus pentoxide. The dried tissue was pulverized to pass a 40-mesh sieve. This powder was extracted with about ten times its weight of dilute NaOH at pH 9.5 for twenty-four hours. After centrifugalization, the residue was extracted for several hours with sufficient water to remove practically all extract not separated from the residue; the mixture was then again centrifugalized. The supernatant fluids were combined and neutralized with dilute hydrochloric acid to pH 7.5, allowed to stand in the icebox overnight, centrifugalized and adjusted in volume so that 1 cc. was equivalent to 100 mgm. of anterior lobe powder.

The other portion of fresh anterior lobe was similarly extracted with dilute sodium hydroxide solution and then partially neutralized (pH 7.5). The concentration of this extract in terms of fresh whole gland was equivalent to 100 mgm. per cubic centimeter or somewhat less than one-fourth that of the acetone desiccated

powder. Some of the comparative experiments performed on these extracts are summarized in table 2. It is apparent that the acetone dessicated gland is a satisfactory source of the oestrus inhibiting principle. The comparison given in this table, however, is not to be considered of great value quantitatively.

The acetone extract obtained above was evaporated to dryness at room temperature and the residue was extracted with dilute NaOH and further treated in the same way as the powdered gland. The concentration of this extract was such that 1 cc. represented 1 gram of original tissue. This solution was injected in 1 cc.

TABLE 2

A comparison of fresh and acetone dehydrated beef anterior lobes as sources of the oestrous inhibiting principle

SOURCE OF EXTRACT	NUM- BER OF RATS IN- JECTED	EQUIVALENT DAILY DOSE		DAYS IN- JECTED	TOTAL NUM- BER OF CYCLES	LENGTH OF CYCLES		<i>t</i>	<i>P</i> *
		Fresh	Dry			Mean	Stand- ard devi- ation		
		<i>mgm.</i>	<i>mgm.</i>			<i>days</i>	<i>days</i>		
Fresh gland.....	6	25		21	16	8.19	± 5.56	2.73	0.02
	6	50		21	6	26.50	± 3.39		
Dried gland.....	6		6.25	21	9	11.56	± 2.24		
	6		12.50	21	9	14.44	± 7.06		
	10		25.00	21	13	19.15	± 9.00		
Acetone-soluble fraction.....	12	1,000		14	42	4.21	± 1.27	0.88	0.37

* The probability that, by random sampling, one would observe a mean oestrous cycle of this length.

doses into 12 rats for fourteen days and was without effect on the oestrous cycles (table 2).

From a stock of desiccated anterior lobe powders other types of extracts have been made and roughly compared in effectiveness with alkaline extracts. An effort was made to standardize the period of injection, but this is more fully discussed later.

If extraction is carried out at an acid pH and the extract is subsequently neutralized, the resulting product is considerably less potent. Acetic acid has been used to lower the pH of the suspension of powder in water to 3.7 to 5.0. The amount of

hormone extracted appears to be particularly small below pH 4.4. Extracts made at pH 4.4 to 5.0 must be administered in two to three times the dose required of an alkaline extract of similar strength. Moreover alkaline extraction of the residue remaining after acid treatment removes most of the unextracted principle.

The oestrus inhibiting principle can be readily extracted by a 50 per cent aqueous pyridine solution (pH ca. 8.9). Such a solution was employed by Fevold, Hisaw and Leonard (9) as a starting point in their method of separating follicle stimulating and luteinizing fractions from pituitary powder. We were unable, in repeating Claus' (10) method for securing from the pituitary a fraction which caused only luteinization, to obtain any oestrus inhibition by such extracts (doses equivalent to 0.75 to 1.0 gram of powder daily).

The oestrus inhibiting principle can be salted out of solution at a neutral or slightly alkaline pH. The only salts employed were sodium sulphate (20 grams to each 100 cc. of solution) and sodium chloride (to saturation).

THE COMPARATIVE OESTRUS INHIBITING AND GONAD STIMULATING PROPERTIES OF EXTRACTS OF SHEEP AND BEEF HYPOPHYSES

Various extraction methods have been employed in this laboratory in comparing the efficacy of sheep undissected pituitary bodies and of beef anterior pituitary glands in stimulating the ovary of the immature rat. Invariably the extracts of sheep glands were much more effective (11). Since beef anterior pituitary was found to be a good source of a principle causing luteinization in the adult rat, it seemed possible that, if gonad stimulation were dependent upon separate follicle maturing and luteinizing principles as contended by Fevold and his co-workers (9), beef glands would contain chiefly the luteinizing hormone and sheep glands would be rich in both principles. Although the experiments of Fevold, Hisaw and Leonard were not confirmed in this laboratory (12), experiments were performed to test the possibility described above.

In the most complete experiment of this group, all extracts were made from the same reagents, in the same concentration, and

injected into the same animals (adults) or their littermates (immatures). Acetone desiccated beef anterior lobe powder and

TABLE 3

The comparative effects of similar extracts of beef anterior pituitary and of sheep whole pituitary

A. On the ovary of the immature rat¹

SOURCE OF EXTRACT	POWDER EQUIVALENT OF TOTAL DOSE	NUMBER OF ANIMALS	MEAN BODY WEIGHT AT DEATH	MEAN OVARIAN WEIGHT AND STANDARD DEVIATION
	<i>mgm.</i>		<i>grams</i>	<i>mgm.</i>
Sheep whole pituitary.....	100	8	40.9	31.40 ± 10.19
Beef anterior pituitary.....	250	8	42.8	22.11 ± 4.27

B. On the oestrous cycles of the mature rat. Daily dose of each preparation equivalent to 15 mgm. Repeated seven times

EXPERIMENT NUMBER	SOURCE OF EXTRACT	GROUP NUMBER	NUMBER OF RATS INJECTED	TOTAL NUMBER OF CYCLES	LENGTH OF CYCLES		SIGNIFICANCE		
					Mean	Standard deviation	Experiments compared	<i>t</i>	<i>P</i> ²
1	Sheep whole pituitary	I	None	32	<i>days</i> 4.22	<i>days</i> ± 0.79	2, 1	4.66	< 0.0001
2		I	20	32	6.94	± 3.20	2, 3	3.95	< 0.0001
3		I	None	73	4.64	± 1.26			
4	Sheep whole pituitary	II	None	82	4.48	± 0.96	5, 4	4.84	< 0.0001
5		II	18	20	7.90	± 3.13	5, 6	4.95	< 0.0001
6		II	None	57	4.33	± 1.33			
7	Beef anterior pituitary	II	None	32	4.00	± 1.14	8, 7	3.20	< 0.002
8		II	20	30	5.43	± 2.18	8, 9	2.31	0.021
9		II	None	82	4.48	± 0.96			
10	Beef anterior pituitary	I	None	73	4.64	± 1.26	11, 10	0.42	0.69
11		I	18	29	4.76	± 1.33	11, 12	0.88	0.37
12		I	None	60	4.50	± 1.24			

¹ Littermate rats were used.

² The probability that a mean oestrous cycle of this length would be encountered in random sampling.

sheep whole pituitary powder were suspended in 0.02 N NaOH, in separate flasks so that the final concentration was 10 grams of powder per 100 cc. of suspension. After seventeen hours' extrac-

tion at room temperature, the pH (glass electrode) of the beef pituitary extract was 7.76 and that of the sheep, 8.13. The residue in each case was separated by centrifugalization and washed three times with equal quantities of 0.001 *N* NaOH, the pH's were adjusted to 7.60 and 7.64 respectively and the final volumes were made equivalent to 5 grams of original powder per 100 cc. of solution. To each solution, after filtration through a Seitz filter, merthiolate was added (final concentration, 0.01 per cent); at the end of the experiment, the solutions were sterile. These solutions as such or diluted were injected subcutaneously into immature or adult rats. The data of this experiment, summarized in table 3, are typical except that extracts of sheep whole pituitary powder commonly caused relatively more oestrus inhibition. Although the extract of beef anterior lobe powder was administered in two and a half times the dose of extract of sheep whole pituitary, yet the latter proved the more potent in bringing about ovarian hypertrophy (table 3, A).⁵ Similarly the extract of sheep whole pituitary was more effective in lengthening the oestrous cycle than the extract of beef anterior lobe (table 3, B). In experiment B, both groups received both extracts. While sheep extract was being administered to group I, beef extract was being administered to group II; the experiment was reversed after the control interval. Two animals in each group became ill in the control interval and were discarded.

From this and similar experiments, we conclude that there is no evidence that beef pituitary glands are richer in a hypothetical luteinizing principle. Such experiments also lend no support to the belief that gonad stimulation is due to the combined action of two principles.

THE SPECIFICITY OF THE OESTRUS INHIBITION CAUSED BY PITUITARY EXTRACT

Detailed control data on the effect of extracts of tissues other than the pituitary are needed. Although both Evans (8) and Claus (10) mention that such experiments have been performed,

⁵ The probability that by random sampling one would observe as great a difference in mean ovarian weights is 0.03.

they give little experimental data. Many believe that the oestrous cycle of the white rat is easily disturbed. However we found this to be the case only when the extract injected (as from adrenal gland and from the posterior lobe of the pituitary) was undoubtedly toxic as was shown by the appearance of the animals and their progressive loss in weight.

In table 4 data on nine other tissues are presented. None caused what we regard as a significant lengthening of the oestrous

TABLE 4

The effect of extracts of tissues other than the anterior lobe on the oestrous cycle of the rat

Daily dose equivalent to 1 gram of fresh tissue

EXTRACT OF	DAYS IN- JECTED	NUMBER OF RATS	TOTAL NUMBER OF CYCLES	LENGTH OF CYCLES		<i>t</i>	<i>P</i>
				Mean	Stand- ard devia- tion		
				<i>days</i>	<i>days</i>		
Thyroid.....	21	10	36	4.47	±1.46	0.32	0.76
Ovary.....	21	10	45	4.82	±2.39	1.19	0.23
Testis.....	21	10	47	4.34	±0.60	0.48	0.61
Liver.....	21	10	46	4.39	±0.85	0	0
Kidney.....	21	10	42	4.21	±0.51	1.89	0.06
Spleen.....	21	10	41	4.71	±0.94	2.05	0.04
Thymus.....	21	10	41	4.59	±1.55	0.81	0.42
Brain.....	21	10	43	4.44	±1.44	0.22	0.82
Muscle.....	21	10	45	4.64	±0.67	2.20	0.03

cycle. The extracts were made from tissues carefully dessicated by acetone, powdered and extracted at an alkaline pH like the pituitary extracts. The dose however was equivalent to 1 gram of fresh tissue per day and was continued over three weeks. These data indicate that the oestrus inhibiting principle is obtained only from the pituitary.

Our anatomical findings also demonstrated that pituitary extracts in comparison with those of other tissues had specific effects on the ovaries. The histological examination of the ovaries of animals treated with anterior lobe extracts revealed few ripe follicles, many corpora lutea and corpora lutea with

enclosed ova. These results are in agreement with those reported by Evans and others. One or two animals of each group receiving extracts of tissues other than the anterior pituitary were sacrificed and their ovaries were examined. As far as possible, those animals having the longer cycles were chosen. The appearance of the ovaries was quite different from those of the animals receiving anterior pituitary; more ripe follicles and fewer corpora lutea were present. The ovaries of the animals injected with extracts of other tissues could be differentiated most easily, however, by

TABLE 5

The effect of different doses of a single preparation on the mean length of the oestrous cycle of groups of rats

Each dose was given once daily for seven days

DOSE		NUMBER OF ANIMALS	OESTRUS CYCLES		<i>t</i>	<i>P</i>	PER CENT OF CYCLES LONGER THAN	
Weight	Relative strength		Number	Mean length and standard deviation			Seven days	Eight days
<i>mgm.</i>				<i>days</i>				
3.1	0.25	40	81	4.93 $\pm$ 1.97	2.46	0.01	7.4	6.2
6.2	0.50	40	86	6.00 $\pm$ 2.90	5.06	<0.0001	25.6	24.4
9.3	0.75	40	58	6.31 $\pm$ 3.09	4.68	<0.0001	31.3	27.6
12.4	1.00	40	53	9.74 $\pm$ 4.82			60.4	58.5
18.6	1.50	20	26	8.38 $\pm$ 4.27			65.5	53.9
24.8	2.00	40	45	10.31 $\pm$ 4.73			69.0	53.3
43.4	3.50	40	43	12.67 $\pm$ 4.94			83.7	81.5
86.8	7.00	40	39	16.15 $\pm$ 5.98			97.5	94.9

the absence of corpora lutea with retained ova. Mounted sections of the ovaries, prepared as unknowns, could easily be recognized by the use of these criteria as those of animals treated either by pituitary extracts or by extracts of other tissues.

THE ASSAY OF THE OESTRUS INHIBITING PRINCIPLE

The data of the most complete experiment on the feasibility of assaying quantitatively the oestrus inhibiting principle are given in table 5. To a large batch of an alkaline extract of beef anterior pituitary warmed to 35°C., sodium sulphate was slowly added and dissolved until the concentration amounted to 20 grams per 100 cc. The precipitate resulting from this procedure

was dissolved in water and the pH adjusted to 7.4. Any undissolved residue was removed by centrifugalization. The clear supernatant solution was passed through a sterile Berkefeld "N" filter and stored in sterile flasks. The final concentration of the solution in terms of beef anterior lobe powder was 100 mgm. per cubic centimeter. The solution was suitably diluted with alkalized saline and administered in a dose of 1 cc. to each animal. Usually 20 animals were injected at one time with each dose. It was thus possible to administer different doses to different groups over the seven weeks required for the experiment. No animal was injected with a new dose until it had exhibited at least two oestrous cycles of normal length.

The data show that as the relative dose is increased, the mean oestrous cycle becomes lengthened and the incidence of long oestrous cycles (greater than seven or eight days) is increased. By such means it is therefore possible to obtain at least crudely quantitative estimates—probably best in comparison with a standard—of the oestrous inhibiting potency of an extract. In this experiment we encountered no *consistently* resistant or susceptible animals. We also found no evidence that our sterile solution underwent deterioration (ca. two months).

SUMMARY

1. Extracts capable of causing the inhibition of oestrus in the adult female white rat were prepared from both beef anterior lobes and from sheep whole pituitary bodies. It was found best to carry out extraction at an alkaline pH. Glands which had been desiccated by acetone and subsequently powdered were found to be satisfactory.

2. Extracts of sheep whole pituitary body (compared with those of beef anterior lobe) were consistently found to be more potent both in stimulating the ovary of the immature rat and in inhibiting oestrus in the adult rat.

3. Extracts of nine control tissues caused no lengthening of the oestrous cycle. The characteristic ovarian changes found in the animals receiving anterior pituitary extracts were not observed in the ovaries of animals receiving extracts of other tissues.

4. The assay of extracts causing the inhibition of oestrus is discussed.

Dr. David Klein of the Wilson Laboratories kindly supplied the beef and sheep pituitary glands used in this study.

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